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Mini review

Steric and electronic properties of the cofactor's amino group control the lifetime of the central carbanion/enamine intermediate in transketolase

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ABSTRACT

Transketolase (TK), a thiamin diphosphate (ThDP) dependent enzyme, catalyzes the reversible transfer of a two-carbon unit from keto- to aldo-substrates. Dihydroxyethylthiamin diphosphate (DHEThDP), formed as a result of cleavage of the donor substrate, serves as an intermediate of the TK reaction. TK from the yeast *Saccharomyces cerevisiae* is unique among thiamin enzymes displaying enzymatic activity after reconstitution with a methylated analogue of the native cofactor, 4′-methylamino-ThDP. The reconstitution of the apoenzyme with both ThDP and the methylated analogue can be analyzed by near UV circular dichroism. It was demonstrated that in the native holoenzyme and in the complex of TK with 4′-methylamino-ThDP the formation of the dihydroxyethyl-based carbanion/enamine took place with comparable rate constants, whereas the protonation of the reactive species was much faster in the complex with the analogue. The enzymatic activity of the enzyme reconstituted with 4′-methylamino-ThDP was 10*fold* higher in the ferricyanide assay. We suggest that a methylation of the 4′-amino group of ThDP impairs the resonance stabilization of the carbanion/enamine intermediate both sterically and electronically, thus allowing either a faster protonation or oxidation reaction by ferricyanide. The formation of the optically active DHE-4′-methylamino-ThDP was monitored by near UV circular dichroism spectra and corroborated by ^{1}H NMR analysis. The protonated form of the intermediate DHE-4′-methylamino-ThDP was released from the active sites of TK and accumulated in the medium on preparative scale.

Transketolase (EC 2.2.1.1) catalyzes the reversible transfer of a two-carbon residue (glycol aldehyde) from keto- to aldo-substrates. The enzyme has a broad substrate specificity. Donor substrates are, for instance: D-xylulose 5-phosphate (X5P), fructose 6-phosphate, sedoheptulose 7-phosphate, erythrulose. Hydroxypyruvic acid (HPA) as donor substrate allows a practically irreversible product formation as carbon dioxide is released. Acceptor substrates are, for instance: D-ribose 5-phosphate (R5P), erythrose 4-phosphate, glyceraldehyde 3-phosphate, glycolaldehyde [1–4].

Transketolase (TK) from the yeast *Saccharomyces cerevisiae* is a well investigated enzyme with known three-dimensional and active centre structure. TK was the first ThDP-dependent enzyme the crystal structure of which has been solved and revealed the general fold for this class of enzymes and the interactions of the non-covalently bound cofactor ThDP with the protein component. It is composed of two identical subunits, each with a molecular mass of 74.2 kDa, with two active centers. The native holoenzyme contains Ca2+. ThDP is bound at the interface between the subunits, interacting with residues from both subunits. Thus, a dimer may be considered as the catalytically competent unit. The coenzyme binding site is, except for the C2-atom of the thiazolium ring, totally inaccessible for the outer solvent [5–7].

Over the last 20 years, many of the important details of TK catalysis could be elucidated by means of X-ray crystallography and detailed kinetic and mutant studies [8–13]. Based on a rational cofactor design ThDP analogues are useful probes for enzyme function. In this context, chemically synthesized cofactor analogues have been utilized as valuable mechanistic probes for studying details of enzymatic thiamine catalysis [14]. The special role of the 4′-amino group of the pyrimidine moiety of the cofactor in ThDP catalysis was emphasized by SCHELLENBERGER already several

Abbreviations: TK, transketolase; ThDP, thiamin diphosphate; DHEThDP, 2-(α,β-dihydroxyethyl)-thiamin diphosphate; DHE-4′-methylamino-ThDP, 2-(1,2-dihydroxyethyl)-4′-methylamino-thiamin diphosphate; GA, glycolaldehyde; HPA, hydroxypyruvic acid; X5P, D-xylulose 5-phosphate; R5P, D-ribose 5-phosphate.

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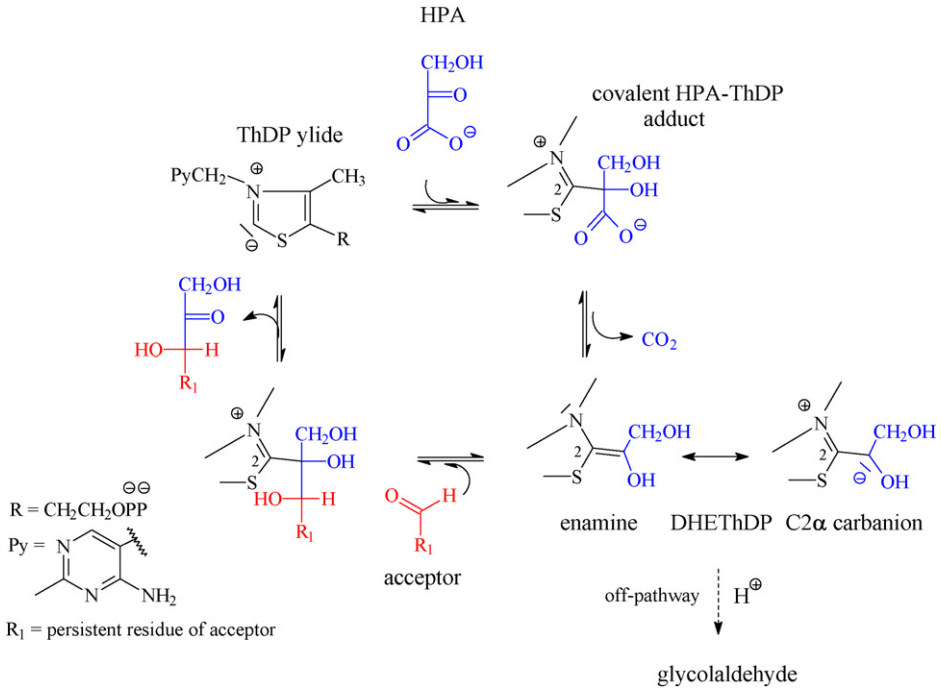


Scheme 1. Catalytic cycle of the transketolase reaction with HPA as donor substrate.

decades ago [14–17]. Later the 4′-amino group of ThDP's aminopyrimidine ring was shown to be essential for deprotonation of the C2 of the enzyme-bound cofactor, the common initial reaction step in all ThDP-dependent enzymes [8,18]. It has been suggested that deprotonation of ThDP's C2 in enzymes is general base-catalyzed by N4′ of the 1′,4′-imino tautomeric form of the cofactor, and assisted by a hydrogen-bonding interaction of a strictly conserved glutamate side chain and N1′ of the aminopyrimidine [7,8,18–20].

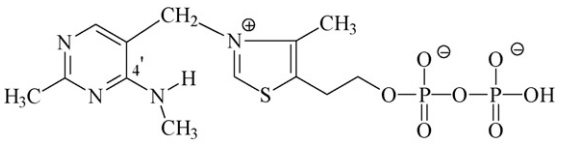
The two-carbon transfer reaction catalyzed by TK can be divided into two parts and is illustrated in Scheme 1, which shows the catalytic cycle of the transketolase reaction with HPA as the donor substrate. The first half of the reaction consists of the following steps: binding of donor substrate and formation of a covalent enzyme-substrate complex, cleavage of the donor substrate and formation of a key intermediate, DHEThDP carbanion/enamine, and release of the first product, CO_2. In the second half of the reaction the intermediate interacts with the acceptor—aldose substrate. The two-carbon unit is transferred to the acceptor substrate and the new ketose with its carbon chain extended by two carbon atoms is released. Thus, TK is a typical transferase enzyme, requiring two substrates for catalysis, a two-carbon unit donor and an acceptor, respectively.

However, in the absence of an acceptor substrate the α-carbanion might be subject to protonation at Cα and release of either glycolaldehyde (GA) or DHEThDP from the active centers. The rate of Cα protonation can be anticipated to be very low because of the apparent stabilization of the carbanion/enamine intermediate on the enzyme.

It was demonstrated, that native TK (in complex with ThDP) is able to catalyze a one-substrate carboligation reaction utilising only the donor substrate. Product of this reaction is erythrulose, which is the result of the condensation between two GA residues formed in the course of the cleavage of two donor substrate molecules [21,22]. So, the way the reaction will proceed, critically depends on the presence or absence of an acceptor substrate and on the electronic state of the aminopyrimidine part of the cofactor.

A suitable way for avoiding a rapid protonation of the intermediate might be the stabilization of an enamine-type intermediate through intramolecular hydrogen-bonding interactions between the 4′-amino group and the dihydroxyethyl part of the intermediate. Indeed, the X-ray crystal structural analysis of the DHEThDP carbanion/enamine intermediate in the active site of transketolase has shown a network of hydrogen bonds in its stabilization and shows interaction between the 4′-amino group and the dihydroxyethyl part of the intermediate. Also, the calculated electron density maps clearly favoured a planar enamine with an sp^2 hybridized Cα atom rather than a pyramidal localized carbanion [23].

In recent publications the methylated analogue of the native cofactor, 4′-methylamino-ThDP (Scheme 2) was used to investigate the function of the aminopyrimidine moiety of the coenzyme in transketolase catalysis, in the formation and stability of the key intermediate of transketolase reaction [24,25]. Early on, this cofactor analogue in the active centre of TK was demonstrated to be coenzymatically active in the carboligation reaction of HPA (donor substrate) and GA (acceptor substrate) yielding erythrulose as a product, and in an artificial redox reaction using ferricyanide that traps the central carbanion/enamine intermediate in an oxidative manner, to give glycolic acid [26]. 4′-Methylamino-ThDP is capable to form the imino tautomer on the enzyme similar to the native cofactor, the basicity of N4′ can be anticipated to be slightly increased by the methyl substituent (positive inductive effect). While using an NMR-based H/D exchange technique, the C2 deprotonation rate constant of the analogue bound to TK was estimated and shown to be virtually identical to that determined for TK in complex with ThDP, clearly demonstrating that only one proton is engaged in the transition state of cofactor activation (N4′-catalyzed C2 deprotonation) [24].



Scheme 2. Structure of 4′-methylamino-ThDP.

$$DHEThDP^- + 2Fe(CN)_6^{3-} + H_2O \xrightarrow{TK} ThDP^- + CH_2OH-COOH + 2Fe(CN)_6^{4-} + 2H^+$$

Scheme 3. The key intermediate DHEThDP is oxidized by ferricyanide yielding glycolic acid.

Table 1
Catalytic parameters of transketolase reaction with ThDP and 4′-methylamino-ThDP (The experimental error upon determination of the above parameters is within 10–15%.).

Cofactor	K_d (μM)	U (mg)[a]	U (mg)[b]	K_m for HPA (μM)[b]
ThDP	0.03[c]; 0.25[c]	37	0.75	70
4′-Methylamino-ThDP	14[d]	0.52	6	7000

[a] The determination of the enzymatic activity was performed in the two-substrate reaction using xylulose 5-phosphate as donor and ribose 5-phosphate as acceptor substrates.
[b] The determination of the enzymatic activity was performed using HPA as donor substrate and ferricyanide as oxidizing agent.
[c] Data from [34].
[d] Data from [26].

1. Crystal structure analyses

Crystal structure analyses have shown that 4′-methylamino-ThDP binds to TK in very much the same way as ThDP. While the electron density for the ThDP moiety of the analogue is well defined no electron density was found for the methyl group. This indicates a high flexibility (rotation) of the 4′-amino nitrogen. There is no amino acid residue in the active site of TK close enough to the 4′-amino group of ThDP for hydrogen bond formation to "freeze out" one conformation only [24].

2. Catalytic activities of TK complexes

The catalytic activities of TK complexes were determined by two different assays: (**1**) the assay detecting glyceraldehyde 3-phosphate using X5P as donor and R5P as acceptor [4] (**2**) the assay measuring the oxidation of the α-carbanion/enamine form of DHEThDP using HPA as a donor substrate and ferricyanide as oxidation agent [27] (Scheme 3).

The kinetic parameters of TK determined by these methods are summarized in the Table 1. Methylation of the cofactor leads to a lower affinity of TK both for the 4′-methylamino-ThDP cofactor and HPA. The specific activity of the enzyme reconstituted with the analogue determined by assay (**1**) was much lower in comparison to the native holoenzyme. In contrast, the enzymatic activity measured by assay (**2**) for the enzyme reconstituted with 4′-methylamino-ThDP was by one order of magnitude higher than for the native holoenzyme.

3. Near UV circular dichroism spectra

Our investigation was based on the induced optical activity of TK upon binding of ThDP and substrates. The interaction of apoTK with ThDP and the formation of catalytically active holoenzyme is accompanied by the appearance of a new band in the CD spectrum (the range 300–380 nm, Fig. 1, curve 2), not present in the spectra of the single components. There is a clear-cut correlation between the quantity of ThDP bound to the apoenzyme and the catalytic activity [29,30]. The appearance of the new absorption band is characteristic for a catalytically active holoenzyme, its intensity would change after TK interaction with its substrates. The addition of HPA to holoTK leads to the formation of the key reaction intermediate, DHEThDP. Formation of the intermediate is accompanied by the inversion of the absorption band in CD spectrum (Fig. 1, curve 3). Recording a spectrum after 20 min revealed no further change of amplitude. A subsequent addition of acceptor substrate GA leads to formation of the second transketolase reaction product and the restoration of the initial holoenzyme and the respective CD spectrum (Fig. 1, curve 4). The formation of the reaction product erythrulose is indicated by the increase in the positive ellipticity between 260 and 300 nm [28]. So, the interaction of the substrates with holoTK and their subsequent conversion is accompanied by significant changes in the intensity of the new absorption band, and these changes are widely used for characterization of individual steps of the TK reaction [22,23,31,32]. Thus, CD spectroscopy is a suitable tool to detect both the donor and acceptor half-reactions independently.

After reconstitution of the apoenzyme with 4′-methylamino-ThDP, the typical near UV CD band of the complex develops, showing a negative extremum (Fig. 2, curves 1 and 2) that is red-shifted by approximately 20 nm when compared to TK in complex with ThDP. The addition of the donor substrate HPA and the subsequent addition of the acceptor substrate GA results in changes of the spectra (Fig. 2, curves 3 and 4), that correspond in principle to these observed for the native holoenzyme. Hence, both steps of the transketolase reaction (carbanion/enamine formation (curve 3) and transfer of the 2-carbon unit to the acceptor (curve 4)) can be analyzed in a similar manner. So, the complex of TK with the analogue is also able to perform both stages of the reaction.

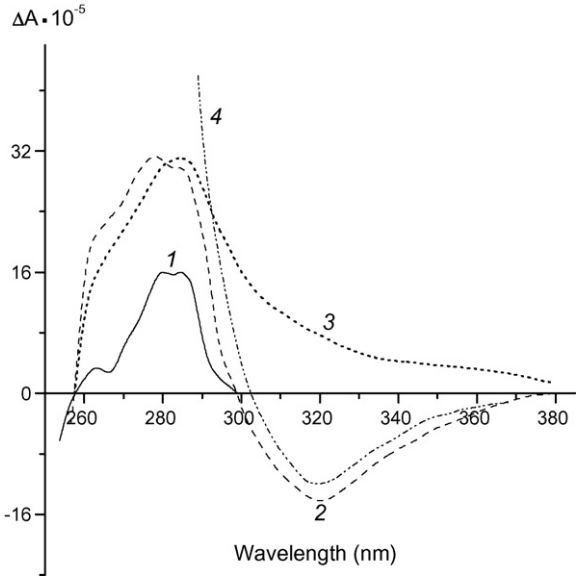


Fig. 1. Near UV CD spectra of transketolase reconstituted with ThDP and substrates: 1—apotransketolase, 2—holotransketolase, 3—holotransketolase + HPA, 4—holotransketolase + HPA + GA [25]. Glycyl-glycine buffer, 50 mM, pH 7.6; $CaCl_2$, 2.5 mM; ThDP, 30 μM; HPA, 2 mM; GA, 20 mM; transketolase, 12 μM (active site). The incubation time after addition of the substrate(s) was 5 min. The formation of erythrulose is indicated by the increasing positive ellipticity at around 275 nm [28]. CD spectra were recorded using a Jobin Ivon Mark V (France) dichrograph interfaced with a computer. Temperature was set at 20 °C, the optical pathlength was 1 cm.

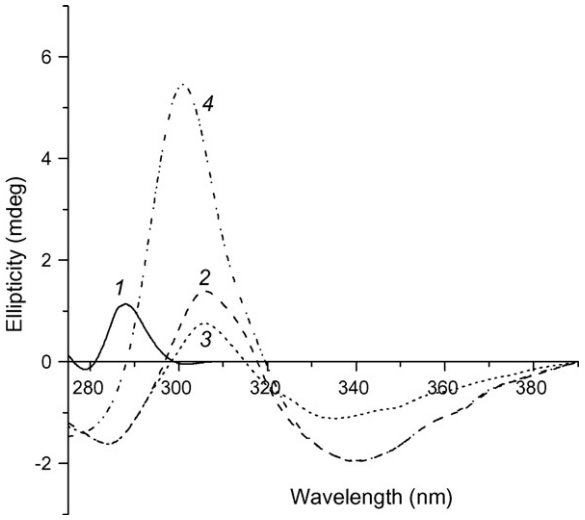


Fig. 2. Near UV CD spectra of transketolase reconstituted with 4′-methylamino-ThDP and substrates: 1—apotransketolase, 2—holoenzyme complex, 3—holoenzyme complex + HPA, 4—holoenzyme complex + HPA + GA [25]. The formation of erythrulose is indicated by the increasing positive ellipticity at around 300 nm. Measurements were performed at a protein concentration of 5.5 μM (active site) in 50 mM glycyl-glycine (pH 7.6) containing 2 mM $CaCl_2$. The apoenzyme was reconstituted with 250 μM 4′-methylamino-ThDP. The concentration of HPA was 3.5 mM, that of GA 10 mM. CD spectra were recorded using a Jasco J810 dichrograph. Temperature was set at 20 °C, the optical pathlength was 1 cm.

4. Kinetics of donor substrate conversion

The most intriguing investigation of the intermediate formation rate and its stability, with ThDP or 4′-methylamino-ThDP as cofactors, were performed using stopped-flow spectroscopy [24]. Addition of the donor substrate HPA to the native holoenzyme (with ThDP) leads to the absorbance change at around 300 nm [33]. Fig. 3 shows the difference absorption spectra of holoTK in the absence (1) and presence of HPA (2) with respect to apotransketolase, ThDP and HPA. Thus, the conversion of HPA can be followed using changes in the absorbance at 300 nm by stopped-flow kinetics.

Progress curves were monitored under *single-turnover* conditions (equal concentration of HPA and the holoTK active sites) (Fig. 4). Two phases in the absorbance progress curves could be

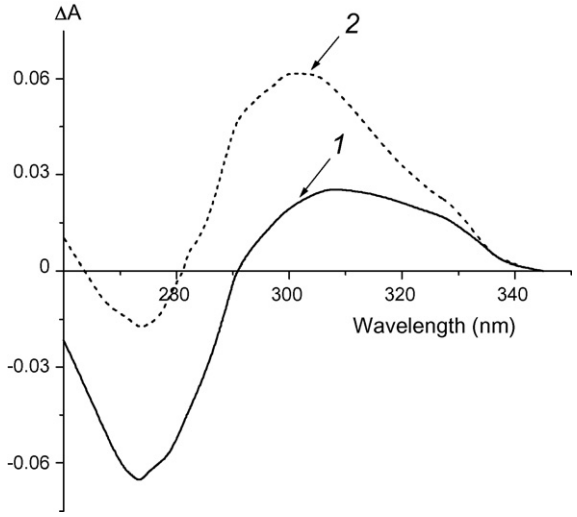


Fig. 3. Difference absorption spectra of holotransketolase in the absence (1) and presence of HPA (2) with respect to apotransketolase, ThDP and HPA. Glycyl-glycine buffer, 50 mM, pH 7.6; $CaCl_2$, 2.5 mM; ThDP, 40 μM; HPA, 2 mM; glycolaldehyde, 20 mM; transketolase, 3 μM.

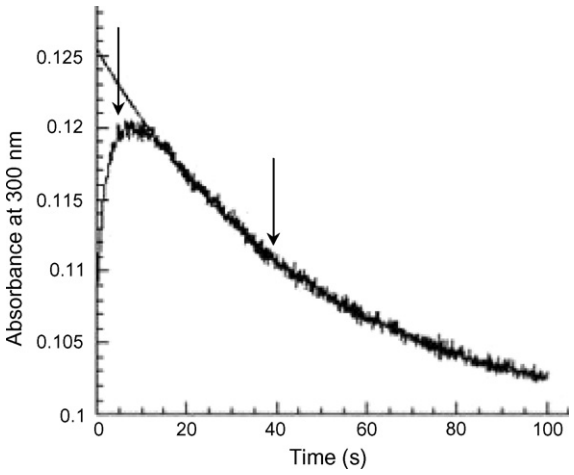


Fig. 4. Stopped-flow kinetics of HPA conversion (one-substrate reaction) by holotransketolase monitored by change in the absorbance at 300 nm [24]. The progress curve was recorded under *single-turnover* conditions. The measurement was carried out at equal protein (active site) and HPA concentrations of 13.4 μM in 25 mM glycyl-glycine (pH 7.6) containing 2 mM $CaCl_2$ and 50 μM ThDP. The temperature was set at 25 °C, the optical pathlength was 1 cm.

observed, a first increase of the absorption represents the key intermediate, DHEThDP, formation, reaching a maximum after 8 s, followed by a subsequent decrease. Each part of this curve can be fitted separately to a single exponential first-order reaction. The rate constant of the second phase is independent of the HPA concentration and was determined to 0.02 s^{-1}. The observed rate constant of the first phase increases with increasing HPA concentration and reaches a maximum value (about 45 s^{-1} at 4 mM HPA).

The conversion of HPA by TK reconstituted with 4′-methylamino-ThDP displayed a maximum change in the absorbance at 320 nm, we could follow it by stopped-flow kinetics also. In these experiments the concentration of HPA was set much above the active site concentration due to the high K_m value of 7 mM (Table 1). Fig. 5 shows the kinetics of donor substrate conversion at 5 mM HPA. The progress curve of the reaction is also characterized by two phases. After an initial increase (first phase) the absorbance decreased (second phase) in a similar way as observed for the native holoenzyme. At longer time scale the absorbance increased again and may be attributed to the

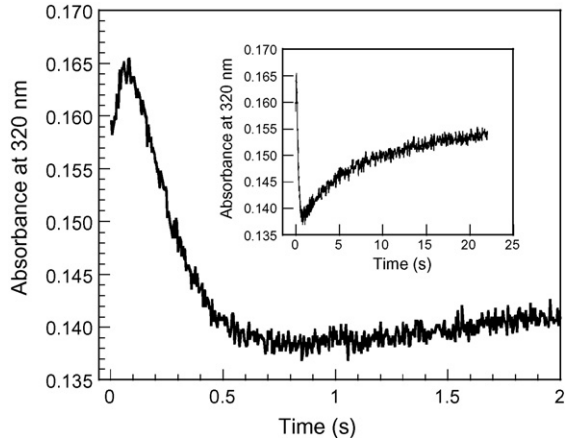


Fig. 5. Stopped-flow kinetics of HPA conversion (one-substrate reaction) by holotransketolase reconstituted with 4′-methylamino-ThDP [24]. The progress curve was monitored by change in the absorbance at 320 nm. The measurement was carried out at a protein concentration of 37 μM (active site) and a HPA concentration of 5 mM in 25 mM glycyl-glycine (pH 7.6) containing 2 mM $CaCl_2$ and 200 μM 4′-methylamino-ThDP. The temperature was set at 25 °C, the optical pathlength was 1 cm. *Inset:* extended progress curve of HPA conversion.

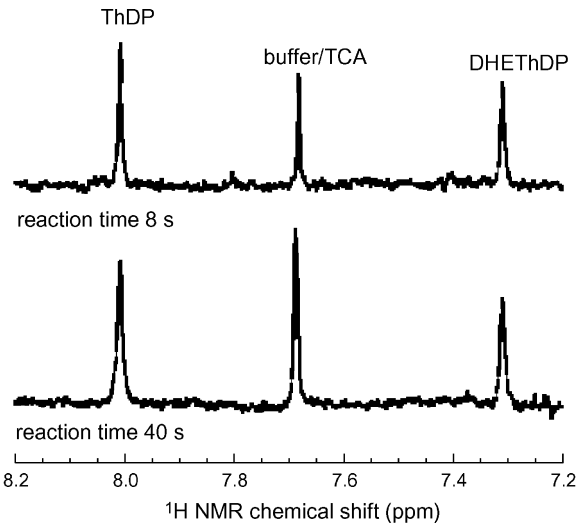


Fig. 6. Intermediate analysis of the donor substrate conversion (one-substrate reaction) holotransketolase under *single-turnover* conditions [24]. The holoenzyme (219 μM active site) was first incubated with 3 mM Mg^{2+} and 219 μM ThDP in 20 mM potassium phosphate (pH 7.6) to allow reconstitution for 5 min at 25 °C. Afterwards the mixture was incubated with the donor substrate HPA at equal concentration giving a final concentration of 109.5 μM. According to the progress curve shown in Fig. 4 the reaction was stopped after 8 s and 40 s by acid quenching, respectively (indicated with arrows). Sample handling, ^{1}H NMR measurements and data analysis were performed according to the method established by TITTMANN [19].

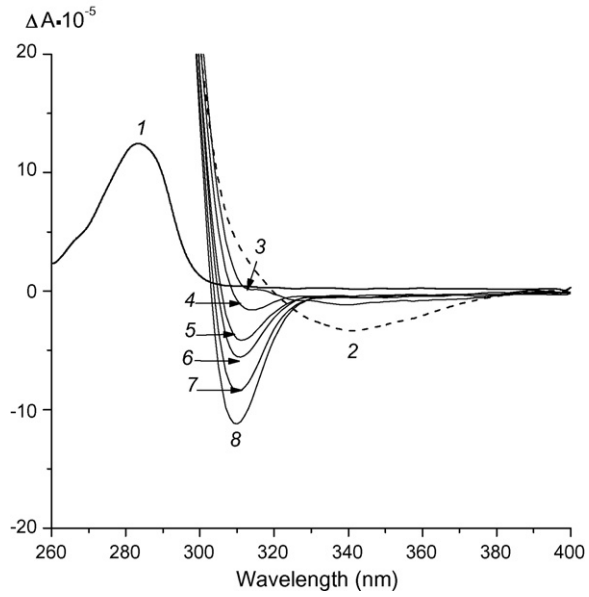


Fig. 7. Near UV CD spectra of transketolase reconstituted with 4′-methylamino-ThDP in the presence of HPA: 1—apotransketolase, 2—holoenzyme complex, 3—holoenzyme complex after addition of the donor substrate HPA, 4–8—time-dependent incease in the CD absorbance recorded with an interscan delay of 5 min [25]. Measurements were performed at a protein concentration of 9.5 μM (active site) in 50 mM glycyl-glycine (pH 7.6) containing 2.5 mM $CaCl_2$. Reconstitution of the apoenzyme was initiated with 250 μM 4′-methylamino-ThDP. The concentration of HPA was 10 mM. CD spectra were recorded using a Jobin Ivon Mark V (France) dichrograph interfaced with a computer. Temperature was set at 20 °C, the optical pathlength was 1 cm.

product formation. A comparison of the kinetic phases in the HPA conversion of the native holoenzyme and of the complex of TK with 4′-methylamino-ThDP monitored by absorbance provides a similar kinetic parameter for the first increase (saturation condition), but a higher rate constant for the second decrease in case of the complex with the analogue. The second phase of the reaction was fitted to a single exponential first-order reaction providing a rate constant of 6 s^{-1} and is found to be independent of the donor concentration.

5. Intermediate analysis

In order to assign the phases of the transient kinetics measured under *single-turnover* conditions mentioned above to distinct intermediates occurring in this reaction, holoTK was incubated with HPA in an equimolar ratio and the reaction stopped after 8 s (maximum amplitude of the absorbance) and 40 s (second phase) by acid quenching (Fig. 4, indicated with arrows). In both cases only the covalent intermediate DHEThDP could be detected by ^{1}H NMR (Fig. 6) according to the method established by TITTMANN [19].

The ^{1}H NMR intermediate method used cannot discriminate between the α-carbanion/enamine and its protonated form, only the protonated form can be analyzed due to the acid quenching. In combination with the transient kinetics and the intermediate analysis the increase in the absorbance can be attributed to the formation of the α-carbanion/enamine of DHEThDP, but the subsequent decrease to its protonation and not to the release of GA. In the course of the equimolar HPA conversion only DHEThDP as reaction intermediate could be detected by ^{1}H NMR and time-resolved X-ray crystallography, both at that time where the absorbance at 300 nm is at maximum, and at longer timescale [23].

Thus, it was demonstrated that in the native holoenzyme and in the complex of TK with 4′-methylamino-ThDP the formation of the dihydroxyethyl-based carbanion/enamine took place with comparable rate constants, whereas the protonation of the reactive species was much faster in the complex with the analogue. This difference in the protonation rate indicates a different stabilization of the intermediate in both samples. Interestingly, the enzymatic activity for the enzyme reconstituted with 4′-methylamino-ThDP was 10*fold* higher in the redox ferricyanide assay. We suggest that a methylation of the 4′-amino group of ThDP impairs the resonance stabilization of the carbanion/enamine intermediate both sterically and electronically, thus allowing either a faster protonation or oxidation reaction by ferricyanide. The data presented further are consistent with this proposal.

6. Investigation of the DHE-4′-methylamino-ThDP stability using near UV circular dichroism spectra

Stability of the DHE-4′-methylaminoThDP was investigated *in the absence of the acceptor substrate* (Fig. 7). Curves 1 and 2 represent the CD spectra of apoTK and apoTK in the complex with the analogue. The addition of HPA to the enzyme is accompanied of a disappearance of the negative band at 340 nm (curve 3). Then we recorded CD spectra every 5 min and observed the formation of a sharp band with a negative maximum at 310–312 nm (curves 4–8). The amplitude of the CD signal is increasing in a time-dependent manner. Furthermore, the intensity of the extremum is unchanged after removal of the protein through filtration of the incubation mixture using a Vivaspin 0.5 ml concentrator. This indicates that the observed negative CD band is caused by a chiral reaction product rather than protein-bound intermediates. In a control experiment without protein, no CD absorbance in this spectral region could be detected demonstrating the formation of the chiral product to be enzyme-catalyzed.

7. Analysis of reaction products by ^{1}H NMR

For a reliable identification of the TK-catalyzed chiral reaction product, the reaction of TK (reconstituted with 4′-methylamino-ThDP and Ca^{2+}) with HPA was analyzed by ^{1}H NMR spectroscopy. It should be noted in this concern, that the enzyme was reconstituted with a 6-fold excess of cofactor analogue to ensure full saturation. A

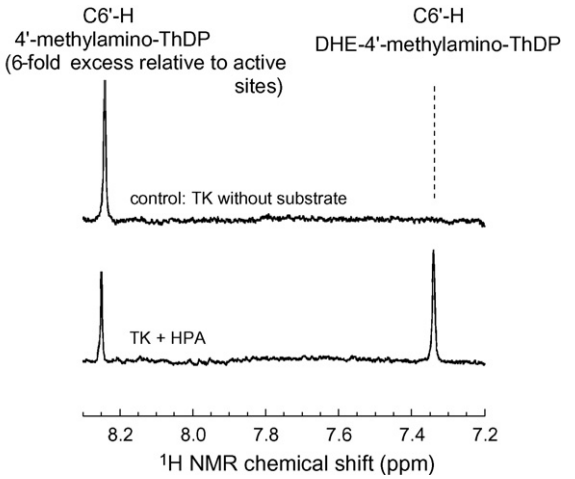


Fig. 8. Analysis of reaction products by ^{1}H NMR spectroscopy [25]. Downfield section of a representative ^{1}H NMR spectrum of an enzymatic reaction mixture after removal of TK. The C6′-H resonances of unreacted cofactor analogue and of the DHE-4′-methylamino-ThDP adduct are indicated.

careful analysis of the reaction mixture by ^{1}H NMR after removal of the enzyme revealed that approximately 60% of the added cofactor analogues were converted to DHE-4′-methylamino-ThDP (Fig. 8). There were no indications that other potential products such as erythrulose had been formed in detectable amounts.

The formation of optically active DHE-4′-methylamino-ThDP was monitored by near UV circular dichroism spectra and corroborated by ^{1}H NMR analysis. The protonated form of the intermediate, DHE-4′-methylamino-ThDP was released from the active sites of TK and accumulated in the medium on preparative scale. The findings suggest that TK in complex with the NH_2-methylated ThDP exhibits a dihydroxyethyl-4′-methylamino-ThDP-synthase activity. Thus, the 4′-amino group of the coenzyme provides kinetic stability of the central transketolase reaction intermediate, dihydroxyethylThDP.

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